THE METABOLISM OF CERTAIN RAPIDLY GROWING HUMAN TUBERCLE BACILLI IN BROTH FREE FROM LIPOIDS AND FATTY SUBSTANCES

STUDIES IN ACID-FAST BACTERIA. II*

A. I. KENDALL, A. A. DAY AND A. W. WALKER

(From the Department of Bacteriology, Northwestern University Medical School, Chicago.)

The study of the metabolism of two rapidly growing, avirulent tubercle bacilli of human origin, in plain, dextrose, mannite, and glycerin nutrient broths, showed a consistent and well-defined progressive breakdown of the protein constituents in all of these media which reached its maximum between the second and the third week (see preceding article). The breakdown of protein was measured by the increase of ammonia. About the third week, ammonia production reached its maximum and then the amount of ammonia, detectable in the media by the method used, showed a definite, progressive recession, so that at the end of five or six weeks the amount of ammonia, altho greater than that in uninoculated controls, was decidedly less than the maximum amount which was found after about three weeks' incubation. No definite explanation for this recession was apparent.

Nutrient broth, as it is usually prepared, contains small amounts of fats, fat derivatives, and lipoids, and it is conceivable that some of these lipoidal substances may play a part in at least the initial phase of this reaction. Lipoids could hardly explain all of the phenomena involved in the ammonia curve, however. Some of these lipoidal substances have attracted considerable attention, particularly in connection with the growth of the tubercle bacillus *in vitro* and in the body. Von Eisler and Laub[1] studied the lipoidal content of the serum of ninety-five tuberculous patients and found it low in all. There was no relation between the decrease of the lipoidal content of the blood and the temperature curve. They found that the cholesterin esters, but not cholesterin itself, were decreased in amount. Deycke and Much[2] and Sieber

* Received for publication July 27, 1914.

1. Wien. klin. Wchnschr., 1913, 26, p. 968.

2. München. med. Wchnschr., 1909, 56, p. 1986; Berl. klin. Wchnschr., 1910, 47, p. 1933; Centralbl. f. Bakteriol., Abt. I, Orig. I, 1910, 54, p. 342.

and Metalnikoff[3] claim that lecithin, neurin, and cholin dissolve tubercle bacilli in the test-tube, while Löwenstein and Beyer[4] deny that the lecithin has any bactericidal action on the tubercle bacillus. These latter observers believe that the liberation of acid, incidental to the decomposition of lecithin, is the cause of the destruction of the organisms.

In order to determine what part, if any, these lipoidal substances, in the amounts in which they occur in ordinary media, might play in the metabolism of tubercle bacilli, media were made up from ingredients in which these substances were definitely and quantitatively eliminated. This was accomplished as follows:

Fifteen grams of Fairchild's peptone were extracted for two weeks with ether, two weeks with alcohol, two weeks with acetone, and ten days with petroleum ether, respectively — in a Soxhlet extractor, the successive extrac-

TABLE 1

METABOLISM OF TUBERCLE BACILLI IN BROTH FREE FROM LIPOIDS AND FATTY SUBSTANCES

Bacillus tuberculosis	Days	Plain Broth A					Plain Broth B				
		Alizarin	Neutral Red	Phenolphthalein	NH_3 mgs. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Alizarin	Neutral Red	Phenolphthalein	NH_3 mgs. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent
597	3	—0.60	—1.00	—0.90	4.2	3.15	—1.40	—1.30	—1.40	11.9	5.32
	7	—1.70	—1.80	—1.30	29.4	22.1	—3.30	—2.80	—1.90	52.5	23.5
	14	—1.90	—1.90	—1.10	38.5	29.0	—3.00	—2.70	—1.70	65.1	29.0
	21	—2.10	—2.10	—1.40	35.0	26.3	—3.80	—3.50	—2.00	60.9	27.2
	28	—1.80	—2.10	—1.10	28.7	21.6	—3.80	—2.90	—1.70	48.3	21.5
W	3	—0.30	—0.50	—0.50	1.4	1.45	—1.40	—1.00	—1.30	11.9	5.3
	7	—1.80	—1.70	—1.30	23.8	17.90	—3.60	—2.90	—1.70	49.7	22.2
	14	—1.60	—1.80	—1.20	37.8	28.4	—2.80	—2.80	—1.80	64.4	28.7
	21	—2.00	—2.20	—1.30	35.0	26.3	—3.70	—3.50	—1.90	60.2	26.9
	28	—1.70	—1.90	—1.30	28.7	21.6	—3.50	—2.80	—1.90	52.5	23.4

tions occurring at intervals of about 12 minutes; these extractions were continued for about six hours daily and for six days per week. Considering the large amount of solvent which bathed this peptone, it is fair to assume that these substances were removed quantitatively. This peptone, which had been extracted, was made into broth by the addition of distilled water; one portion consisting of peptone alone; one portion containing peptone and 1 percent of dextrose; and a third portion containing 3 percent of glycerin in addition to the peptone. Three grams of Na_2HPO_4 and five grams of NaCl per liter were added to each kind of medium. A fourth lot of broth, containing unextracted peptone and the same salts, but with no additional source of carbon, was prepared under the same conditions to serve as a control.

All of the utensils used in the preparation of these fat-free media were cleaned first with alkaline potassium permanganate, then with oxalic acid,

3. Centralbl. f. Bakteriol., Abt. I, Orig., 1910, 54, p. 349.
4. Ibid., 56, p. 160.

then with chromic acid, and washed thoroughly in water and then with distilled water, so that it is certain that no foreign substance could have been introduced into the media during the process of manufacture.

The media thus prepared contained no meat extract or meat juice. It is impossible to rule out the presence of minute traces of dextrose or muscle sugar. These media were distributed in flasks in the usual manner, 100 c.c. per flask, and autoclaved at the same time and inoculated respectively with the two strains of rapidly growing avirulent tubercle bacilli, W. and 597.

The determinations were made in precisely the same manner as those in Study I. The media are designated for purposes of convenience Plain Broth A, Plain Broth B, Dextrose Broth A, and Glycerin Broth A. Plain Broth B, it will be remembered, was made from peptone which had not been extracted. Plain Broth A, Dextrose, and Glycerin Broths were prepared from the peptone which had been extracted as outlined.

Several features in the metabolism of these organisms are noteworthy (Table 1). Plain Broth A (uninoculated) contained 133 mg. of nitrogen per 100 c.c. of broth, while Broth B, which had not been

TABLE 1—(*Continued*)

METABOLISM OF TUBERCLE BACILLI IN BROTH FREE FROM LIPOIDS AND FATTY SUBSTANCES

Bacillus tuberculosis	Days	Dextrose Broth A					Glycerin Broth A				
		Alizarin	Neutral Red	Phenolphthalein	NH_3 mgs. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Alizarin	Neutral Red	Phenolphthalein	NH_3 mgs. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent
597	3	—0.70	—0.70	—1.30	6.3	4.50	—0.50	—0.70	—0.60	2.10	1.58
	7	—.040	—0.40	—1.20	6.3	4.50	0.00	—0.30	—0.60	3.5	2.63
	14	—1.00	—1.00	—1.50	29.4	21.0	0.20	—0.50	—0.80	—0.70	—0.52
	21	—1.50	—1.60	—1.50	23.1	16.5	0.20	—0.60	—0.70	—2.10	—1.58
	28	—1.40	—1.50	—1.40	23.8	17.0	0.20	—0.60	—0.90	—2.80	—2.10
W	3	—0.60	—0.70	—1.20	5.6	4.0	—0.30	—0.40	—0.50	3.5	2.63
	7	—0.60	—0.60	—1.40	1.4	1.0	—0.10	—0.40	—0.80	1.4	1.05
	14	—0.90	—1.50	—1.40	17.5	12.5	0.10	—0.70	—0.80	—2.8	—2.1
	21	—1.50	—1.70	—1.50	23.1	16.5	0.10	—0.60	—0.80	—2.8	—2.1
	28	—1.50	—1.40	—1.40	23.8	17.0	0.10	—0.80	—0.90	—2.8	—2.1

extracted, contained 224 mg. of nitrogen in the same volume. That is to say, during the process of extraction a considerable amount of nitrogenous substance had been removed coincidently with the removal of the fatty substances. The nature of these nitrogenous substances is unknown. In Plain Broth A and Plain Broth B the ammonia production reaches its maximum at the end of the fourteenth day, at which time it is about 29 percent of the total nitrogen of the media. Notwithstanding the fact that the percentage of ammonia to total nitrogen is the same in both media, the actual amount of ammonia in Plain Broth B, that is, the unextracted broth, is much greater, being, roughly, in the proportion of 65 mg. to 38 mg. It would appear that the

removal of certain nitrogenous substances from Plain Broth A did not materially influence the percentage of protein breakdown as compared with Broth B, and the results suggest, furthermore, that the additional nitrogenous content of Broth B was broken down readily. Plain Broth B was distinctly more alkaline in reaction than Plain Broth A, and this increased alkalinity can be explained tentatively on the basis of the greater production of ammonia in this medium. In the dextrose broth, the amount of ammonia produced was distinctly less than that in the corresponding plain broth. This might be interpreted as a sparing action of the dextrose for the protein constituents of the broth, but this sparing action is far less marked, if indeed it be a sparing action, than is the case with ordinary bacteria studied under the same conditions.[5] In glycerin broth, after a slight initial increase in ammonia amounting to about 2 percent of the total nitrogen of the medium, the ammonia appears to decrease in amount, so that at the end of the experiments it is less than that contained in the uninoculated media. At the end of the second week, the glycerin broth cultures of both strains of the tubercle bacillus were found to be slightly viscid, and by the end of the fourth week, this viscosity was very marked. The reaction of the medium to phenolphthalein becomes progressively alkaline in spite of this decrease in ammonia. There is no satisfactory explanation for this phenomenon available at the present time. It is conceivable that at least some of this ammonia is tied up in the bodies of the bacteria, and inasmuch as the organisms studied in this connection form firm tenacious pellicles, leaving the medium beneath them perfectly clear and free from bacteria, it was a comparatively simple matter to make a determination of the total nitrogen of the clear underlying broth. The analyses follow.

Organism	Days	Plain Broth A		Plain Broth B		Dextrose Broth A		Glycerin Broth A	
		Mgs. N per 100 c.c.	Percent Total N Loss	Mgs. N per 100 c.c.	Percent Total N Loss	Mgs. N per 100 c.c.	Percent Total N Loss	Mgs. N per 100 c.c.	Percent Total N Loss
Control	—	133	000	224	000	140	000	133	000
W	28	98	26.3	147	34.3	98	30.0	56	57.9
597	28	77	42.0	154	31.3	96	35.0	42	68.4

5. Kendall, Day and Walker, Jour. Am. Chem. Soc., 1913, 35, p. 1208.

The results show that a very considerable proportion of the total nitrogen in the medium is, apparently, tied up in the bodies of the bacteria. It might be objected at this point that some of this nitrogen may have escaped from the medium, as ammonia, by evaporation, escape perhaps being facilitated by the pellicle floating on the surface. Ammonia, in the amounts produced in these media, almost certainly could not evaporate from the free surface of the medium because of the great affinity of ammonia for water. It is possible, however, that the presence of a pellicle might result in a direct "exhaling" of ammonia into the air, the pellicle acting as a barrier to its reabsorption. This pellicle, it should be remembered, is somewhat dry, and while some loss may have taken place, the amount is probably insignificant when compared with the total amount of nitrogen of the medium. It will be observed that in glycerin broth there was a much greater amount of nitrogen in the pellicle than is the case with the other media. This might be accounted for on the basis of a difference in the luxuriance of the growth, and it is a fact that the pellicles formed, respectively, on Plain Broths A and B are thinner and less extensive than the one formed in glycerin. The pellicle formed in dextrose, however, appears to be quite as dense as that formed in glycerin. It is a noteworthy fact that even the organisms grown in the Plain Broth A, which, theoretically at least, is free from all fats, fatty derivatives, and lipoids, are acid-fast.

While these experiments do not by any means prove that the substance or substances conferring acid-fastness on these organisms are derived from protein derivatives alone, yet it would seem that an experiment of this sort carried out under similar conditions, with especial emphasis on the fat and wax content of the organisms, would throw some definite light on the physiology of the formation of fats and waxes from protein.

The experiments do not explain the recession of ammonia which was noticed in broths containing small amounts of fats and lipoids, and it is probable that these substances do not play any material part in this recession. It is worthy of note that the reaction curve of these organisms in glycerin broth does not conform to the Theobald Smith curve for human tubercle bacilli, the reaction produced being progressively alkaline instead of becoming acid. The composition of the glycerin broth in which these observations were made, however, is so different from that usually employed for this purpose that the results are not at all comparable in the two instances.